

Research Article

Evaluation of the Physicochemical, Antioxidant, and Antibacterial Properties of Tunichrome Released from *Phallusia nigra* Persian Gulf Marine Tunicate

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The aim of this study was to evaluate the physicochemical, nutraceutical, antioxidant, and antibacterial properties of tunichrome released from Persian Gulf tunicate (*Phallusia nigra*). For this purpose, molecular weight (SDS-PAGE), amino acid profile, chemical composition (GC-MS), mineral composition, functional groups (FTIR), total phenol content (TPC), total flavonoid content (TFC), antioxidant activity, and antimicrobial properties were investigated. The results showed that tunichrome contained a high amount of essential amino acids (i.e., Lys = 32.24 mg/100 g) and essential minerals. According to GC-MS results, tunichrome had different antioxidant and antimicrobial components. The TPC and TFC of tunichrome were 0.55 mg GA/g and 0.21 mg quercetin/100 g, respectively. Tunichrome showed higher antioxidant activity than ascorbic acid, and its radical scavenging activity values were increased from 30.28 to 82.08% by increasing concentration from 50 to 200 ppm. Inhibition zones of *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella enterica*, and *Escherichia coli* O157:H7 were 14, 18, 17, and 15 mm, respectively. Moreover, the minimum inhibitory concentration values of tunichrome for *S. aureus*, *Bacillus cereus*, *S. enterica*, and *E. coli* O157:H7 were 1.17, 0.59, 0.59, and 1.17 mg/ml, respectively. The minimum bacterial concentrations were 2.34, 1.17, 1.17, and 2.34 mg/ml for *S. aureus*, *Bacillus cereus*, *S. enterica*, and *E. coli* O157:H7, respectively. These results showed that tunichrome of *Phallusia nigra* has excellent biological effects as a bioactive compound for food fortification.

1. Introduction

Since the synthetic antioxidants and antimicrobial components could exert several side effects, the interest of consumers and producers for safe and natural ingredients is growing [1]. Bioactive compounds are the most attractive ingredients in the design and development of functional foods. The tunicates are a wide marine animal group whose bodies are covered by the cellulose-containing tunic. They are marine filter invertebrates that exhibit the properties of the vertebrates. The accumulation of a high level of metals (i.e., either vanadium or iron) in seawater can be possible by

intracellular polymer matrices [2, 3]. Thus, in the presence of metal chelators such as catechol and pyrogallol groups, tunichromes are associated with metal reduction. Moreover, tunichrome can form covalent crosslinking interactions or complexes with different types of multivalent ions in seawater. This ability can affect its functional properties such as swelling, solubilization, coagulation, and precipitation behavior.

The ascidians, thaliaceans, and appendicularians are the three main groups of tunicates [4]. These animals are consumed in Asia, Chile, and the Mediterranean in the past. These products come from sea wild and cultured

populations when the demand is high, especially for *Halocynthia* and *Styela* species. Edible species are usually from solitary stolidobranchs. *Halocynthia aurantium*, *H. roretzi*, *Microcosmus hartmeyeri*, *M. sabatieri*, *M. vulgaris*, *Polycarpa pomaria*, *Pyura chilensis*, *Styela clava*, and *S. plicata* are the important species eaten. These groups are presented in fresh and dried forms in the markets. High-quality processes of *H. aurantium*, *H. roretzi*, *P. chilensis*, *S. clava*, and *S. plicata* are the main seafood exports to Europe and America [5].

Generally, tunicates have a high nutritional value due to the presence of a high level of bioactive components and protein and low calories [6–10]. Moreover, some of them contain different vitamins (i.e., vitamin E, vitamin B12, and vitamin C), minerals (i.e., Na, K, Ca, Mg, P, Fe, Zn, and Cu), amino acids, folic acid, fatty acid, and pantothenic acid [11]. Some groups of tunicates have different low molecular weight peptides in their bodies such as styelins, plicatamide, halocyamines, lamellarins, and ferreascidin from *Styela clava*, *Styela plicata*, *Halocynthia roretzi*, *Didemnum chartaceum*, and *Pyura stolonifera*, respectively, as well as tunichromes from different tunicates species [5, 12, 13].

Some tunicates species can accumulate low molecular weight oligopeptides in their blood cell which are known as tunichromes. Tunichromes could play an important role in defense mechanism, mainly due to the phenoloxidase which can attack easily tunichrome. It is considered as a key component for both tunic formation and tunic wound healing which use the same primary biochemical mechanisms [13]. Tunichromes have different colors such as pink, red, and blue [14]. These pigments are ingredients in the blood cells of tunicates. Tunichromes were identified in 11 groups of different tunicates [5]. The broad biological activity of tunichromes remains mainly unknown. However, some researches offer unique functionalities for tunichrome in terms of antioxidant [15–17], antimicrobial [5, 18], and anticancer activities [19].

To the best of our knowledge, there is no information on the biological activities of the amazing tunichrome released from the *Phallusia nigra* Persian Gulf marine tunicate. Therefore, this research is a first attempt to evaluate the physicochemical, nutraceutical, antioxidant, and antibacterial properties of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate.

2. Materials and Methods

2.1. Materials. *Phallusia nigra* tunicate was randomly collected from Nayband Bay situated in the north (27° 30'S, 52° 35'E) of Bushehr, Iran, using SCUBA diving at the depth ranging from 0.5 to 1.5 m in spring of 2019. Media cultures and all other chemical materials were prepared in analytical grade from Merck and Sigma-Aldrich.

2.2. Methods

2.2.1. Sampling and Sample Preparation. 50 samples of *Phallusia nigra* tunicates were washed by double distilled water (DDW) to remove contaminants. They were immediately transported to the laboratory in a cool box (in ice-

cold condition) and kept at room temperature for 30 min to release a pink color solution (tunichrome). The obtained suspension was centrifuged (4000 × g for 5 min) and then filtrated by Whatman No. 1 filter paper. The final solution was freeze-dried and powdered for further studies.

2.2.2. Total Protein Content. Total protein content was measured based on the AOAC method by the Kjeldahl device (Buchi, Switzerland).

2.2.3. Molecular Weight Measurement (SDS-PAGE). For the molecular weight determination, the tunichrome (5 mg/mL) was dissolved in 1% SDS solution prepared by phosphate buffer (pH 7.0) and stirred for 24 h followed by centrifugation at 5000 × g for 6 min at 20°C [20]. The supernatant (20 µL) was added to 10 µL Tris-HCl buffer (10 mM Tris-HCl, 10% (w/v) SDS, 0.1% (w/v) bromophenol blue, and 10% (v/v) glycerol) with or without 2% (v/v) β-mercaptoethanol. The suspension was then heated at 95°C for 11 min, and 20 µL of the suspension was developed on the gel slab which was contained resolving gel (12.5%, pH 8.8) and stacking gel (6%, pH 6.8). PowerPac 1000 (Bio-Rad, USA) was used for running the electrophoresis. The running buffer was made by diluting 100 mL 10 X Tris/Glycine/SDS buffer with the DDW at the constant voltage 220 V. The Coomassie Brilliant Blue was used for staining the gel for 60 min, and water was used for destaining for 24 h. The molecular weights were evaluated by comparison to Sinaclon markers (PR901641, CinnaGen Co., Tehran, Iran) at the ranges of 245–11 kDa.

2.2.4. Amino Acids' Profile. To evaluate amino acid profile in tunichrome, peptide bonds of protein were broken by hydrolysis. The hydrolysis process was conducted by heating the sample in an oxygen-free condition containing 6 M HCl and 0.1% phenolphthalein at 110°C overnight. Amino acids were measured by a Varian chromatographic system, containing a 1525 pump, a 9100 autoinjector, and a UV-vis detector. The hydrolyze suspension was injected into an automatic precolumn reaction by 0.1 mL of derivatizing reagent. This chromatographic process contained a solvent mixture (PBS buffer (10 mM, pH 4): acetonitrile (25:75) at a flow rate of 1 mL/min. In this system, a C18 Waters Nova-Pack reverse phase column (particle size 5 µm, 250 × 4.6 mm internal diameter) was applied. All the chromatographic data were processed in a V. 4.5 Star workstation supplied by Varian [21].

2.2.5. Na, Ca, K, Mg, Mn, Cu, Fe, and Zn Measurements. The concentrations of Na, Ca, K, Mg, Mn, Cu, Fe, and Zn of tunichrome were analyzed by a Selectra 2 auto-analyzer (Vital Scientific, Spankeren, Netherlands). To this end, 1 g of lyophilized powder was dissolved in the 5 g of DDW for 24 h and then centrifuged.

2.2.6. Heavy Metal (Cd, As, Pb, and Hg) Measurements. The lyophilized powder of tunichrome (0.2 g) was weighed and mixed with concentrated HNO₃ (7 mL, 65% v/v) and

H₂O₂ (1 mL, 30% v/v) in the polytetrafluoroethylene vessel. The vessel temperature was raised to 160°C for 21 min by mixing at 1500 W in the magnetron (ETHOS One, Milestone, Italy). After this digestion process, the sample was cooled for 120 min, and then, the final volume of the sample reached 50 mL with DDW. The determination of heavy metals was conducted by an atomic absorption spectrometry (Atomic Absorption Spectrophotometer, Varian AA240 FS) system which contained an electrode discharge lamp for evaluating volatile and nonvolatile toxic heavy metals by using argon gas. A graphite furnace was applied to determine nonvolatile compounds (Pb and Cd), and a flow injection-mercury hydride system was applied to calculate volatile (As and Hg) compounds.

2.2.7. Fluoride, Chloride, Bromide, Nitrite, Nitrate, Phosphate, and Sulfate Content Measurement. This measurement was done by an ion chromatograph 761 Compact IC (Metrohm, Herisau, Switzerland) with anion self-regenerating suppressor Metrohm Suppressor Module MSM and conductivity detector. Anion separation was performed by a “Star-Ion-A300” column (100 mm × 4.60 mm, Phenomenex, Torrance, USA). A “Metrosep A PCC 1 HC” column (12.5 mm × 4.0 mm, Metrohm, Herisau, Switzerland) was applied for preconcentration. The volume sample loop was 20 µL. Sample injection to the ion chromatography was done by a 5 mL Becton Dickinson syringe (Fraga, Spain) [22].

2.2.8. FTIR Spectroscopy. FTIR spectroscopy (WQF-510 FTIR Rayleigh, Beijing Rayleigh, China) was used for the evaluation of the peak absorbance intensity of the functional groups in the structure of tunichrome. To this end, dried powder of tunichrome was mixed with KBr to form a compact plate. The FTIR spectra were recorded in the wavenumber range of 4000–400 cm⁻¹.

2.2.9. Determination of Chemical Composition by GC-MS. The chemical composition of tunichrome was investigated by a GC (7890B, Agilent Technologies, Santa Clara, CA, United States) system containing MS (5977A Network, Agilent Technologies). Briefly, 200 mg of tunichrome was homogenized with 200 mL of methanol: chloroform: hexane (1 : 1 : 1), for 24 h in 200 rpm and then centrifuged for 20 min at 4000 rpm. The final supernatant was used for evaluating the chemical composition. The GC system was equipped with an HP5 MS column (nonpolar column, Agilent Technologies, internal diameter: 30 m × 250 µm, film thickness: 0.25 µm). The flow rate of carrier gas (Helium) was 1 mL/min. The temperature of the injector was set at 120°C. The oven temperature program included three steps: (1) 50°C for 1 min, (2) temperature raising to 300°C at a rate of 15°C/min, and (3) holding at 300°C for 20 min. The total processing time was 37.66 min. The scanning range of mass spectra was 50–550 m/z in the EI mode at 70 eV. Components were identified based on mass spectra in comparison with those deposited in the database of NIST11 (U.S.

Department of Commerce, Gaithersburg, MD, United States) and literature data [23].

2.2.10. Antioxidant Properties

Antioxidant Activity. The radical scavenging activity (RSA) (%) was determined by the DPPH° radical scavenging method according to the technique of Ruengdech and Siripatrawan [24]. First, 200 mg of tunichrome with different concentrations within the range of 50, 100, 150, and 200 µg/mL was mixed with 800 µL methanol. Then, 400 µL of each diluted tunichrome was homogenized with 1.6 mL of DPPH° solution (0.1 mM). The suspension was kept at 25°C for 1 h under dark conditions, and finally, the absorbance of the suspension was recorded at 517 nm. A sample without tunichrome was used as a control. The RSA (%) was determined based on the following equation:

$$\text{RSA (\%)} = \left[\frac{(A_c - A_s)}{A_c} \right] \times 100, \quad (1)$$

where A_c and A_s express the absorbances of control and test samples, respectively.

Total Phenol Content (TPC). The TPC was determined by Folin-Ciocalteu reagent assay based on the method of Majzoobi et al. [25]. In brief, samples were mixed with 750 µL of 10% w/w Folin-Ciocalteu (diluted in DDW) and then held at 20°C for 15 min. Afterward, 750 µL of 0.2% sodium carbonate was mixed with the suspension, and the absorbance of the sample was determined at 765 nm after 1 h incubation in a dark place. The TPC was calculated via a calibration curve of different concentrations of a gallic acid solution (0 to 150 µg/mL) and reported as mg gallic acid/g of tunichrome weight.

Total Flavonoid Content (TFC). The method developed by Bagheri et al. [26] was used to measure TFC. For this purpose, 0.5 g of samples and 500 µL of methanol were homogenized with 100 µL of AlCl₃ (10% w/v), 100 µL of CH₃CO₂K (1 M), and 2800 µL of DDW. Absorbance at 415 nm was measured after incubation at 23°C for 35 min. The standard calibration curve was plotted at the same wavelength for various quercetin concentrations (5 to 30 µg/mL methanol). The TFC was reported as mg quercetin/g of the sample weight.

2.2.11. Antimicrobial Properties

Well Diffusion Agar Method. The tunichrome sample antimicrobial activity was measured against selected Gram-positive and Gram-negative bacteria including *Bacillus cereus* (ATCC 11778), *Staphylococcus aureus* (ATCC 6538), *Salmonella enterica* (ATCC14028), and *Escherichia coli* (ATCC 35218) by well diffusion agar method. Muller Hinton agar plates were cultured using 0.1 mL of bacterial suspension with a cell density of 0.5 McFarland standard ($\approx 1.5 \times 10^8$ CFU/mL). Wells with 4 mm diameter were

created by a sterile punch and filled with 50 μ l of the sample (150 mg/ml). After 24 h incubation at 37°C, the diameter inhibition zone (DIZ) was determined in mm.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Method. The broth microdilution technique was used for the evaluation of MIC. First, broth subcultures were produced by inoculating one colony of each bacteria grown 24 h in the 50 ml flask with 20 ml Mueller Hinton Broth in a shaker incubator (Jal, Tehran, Iran) at 150 rpm. Then, the final concentration of bacterium was set at 1.5×10^6 CFU/ml followed by separating the cells using centrifugation at $6000 \times g$ for 5 min. Then, the pellet was dispersed in sterile saline (0.9% NaCl) and was applied for inoculation by 96-well microplates with tunichrome serial dilutions from 0 to 150 mg/ml. The microplates were held at 37°C overnight. After incubation, bacterial growth was determined by turbidity methods. The concentration around MIC was cultured on the MRS agar for determining the MBC.

2.2.12. Statistical Analysis. All data were determined in triplicate. Analysis of variance (ANOVA), and the Duncan multiple range tests were done to evaluate the significant differences ($P < 0.05$) among the average values (SAS ver. 9.1, 2002–2003 by SAS Institute Inc., Cary, NC, USA).

3. Results and Discussion

3.1. Molecular Weight Measurement. SDS-PAGE of tunichrome released from Persian Gulf marine tunicate is shown in Figure 1. The bands at 245, 180, 135, 100, and 75, between 75 and 63, between 63 and 48, 48, 35, between 35 and 25, 25, 20, between 20 and 17, 17, between 17 and 11, and 11 kD were identified in the sample. Two main bands were between 63 and 48 and 20 kD. This result showed that there were different proteins and peptides with antioxidant and antimicrobial properties in the tunichrome.

3.2. Amino Acids' Profile. The protein content of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate was $0.7 \pm 0.02\%$. Amino acids' profile of tunichrome by HPLC is reported in Table 1. The main amino acids were lysine, arginine, glycine, aspartic acid, and proline with concentrations of 32.24, 14.34, 12.44, 10.62, and 9.47 mg/100 g, respectively. Therefore, tunichrome can be considered as a suitable source of lysine essential amino acid (EAA). Kumaran and Bragadeeswaran [27] reported that the release of *E. viride* is containing leucine (582.3 μ g/g), arginine (365.4 μ g/g), lysine (344.5 μ g/g), threonine (295.6 μ g/g), and isoleucine (231.2 μ g/g) and the release of *D. psammathodes* contains leucine (540.9 μ g/g), arginine (401.2 μ g/g), lysine (385.4 μ g/g), threonine (312.5 μ g/g), and isoleucine (254.1 μ g/g). Karthikeyan et al. [28] evaluated the amino acid profile of solitary ascidian *Microcosmus exasperatus* and reported the presence of a total of seventeen essential and nonessential amino acids (nEAA). Among them, ten

essential and seven nonessential amino acids were reported in ascidians mussel. The maximum recorded level of the essential amino acid (567.3 mg) was leucine, and the least level of nonessential amino acids (0.212 mg) was aspartic acid. Tabakaeva and Tabakaev [29] also evaluated the different amino acids of the ascidian *Halocynthia aurantium* in the Japan Sea. Among eighteen identified amino acids, eight of them were essential and the others were nonessential. Internal organs had the highest essential amino acid (50.61%) content and tunic had the lowest (35.01%) content. The common nonessential amino acid in all parts (5.84–10.16%) was aspartic acid.

The contents of EAA, semiessential amino acids (SEAA), and nEAA of tunichrome released from Persian Gulf marine tunicate are reported in Figure 2. It contained 44.4, 21.3, and 34% of EAA, SEAA, and NEAA, respectively. Cho et al. [7] and Kang et al. [10] reported that the protein content and amino acid type are related to the age and type of tunicate, environmental condition, pH of water, and the salt type and concentration.

3.3. Mineral Content. Table 2 shows the Na, K, Mg, Ca, Mn, Zn, Fe, and Cu contents of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate. The main cation ions of tunichrome were sodium, potassium, and magnesium with concentrations of 858.4, 778.5, and 94.7 mg/100 g, respectively. Based on these results, this sample is a good source of essential minerals for the human body. Lee et al. [30] also reported that the internal part of tunicate from Korea was containing Na, K, Mg, Ca, Mn, Zn, Fe, and Cu, in the range of 1471.1–1257.9, 39–98.3, 42.8–78, 129.1–273.3, 5.2–0.9, 0.2–0.4, 2.5–0.9, and 0–0.2 mg/100 g, respectively. Papadopoulou and Kanas [31] reported that Zn and Fe of tunic of *Ciona intestinalis* were 110 and 610 mg/kg. Cho et al. [7] reported that the mineral type is related to the age and type of tunicate, environmental condition, and pollution.

Table 3 shows the heavy metals of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate. This source contained high amounts of heavy metals such as Pb and Hg with concentrations of 36.35 and 8 μ g/g, respectively. The main idea is culturing this tunicate in the pure water instead of the sea. Lee et al. [30] evaluated the internal part of tunicate from Korea and reported a trace content of chromium, lead, silver, and arsenic heavy metals. Papadopoulou and Kanas [31] reported 1, 1.9, 0.011, 0.021, 0.041, 3.7, 0.44, and 0.15 ppm of Se, Cr, Ag, Cs, Sc, Rb, Co, and Sb for the tunic of *Ciona intestinalis*, respectively.

Table 4 shows the concentrations of fluoride, chloride, bromide, nitrite, nitrate, phosphate, and sulfate of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate. Chloride (1750 μ g/g), nitrate (850 μ g/g), and fluoride (17.50 μ g/g) were the main anions of tunichrome. Since a high amount of nitrite and nitrate in the processed food can lead to different cancers, the low content of nitrite and nitrate in tunichrome showed its beneficial health effects [32].

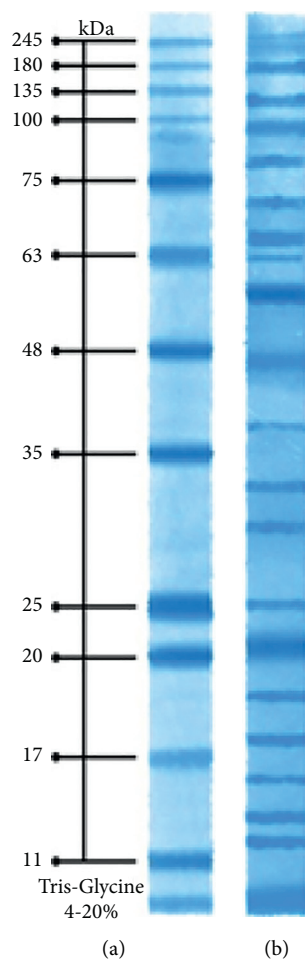


FIGURE 1: SDS-PAGE: (a) sinclon marker (Tris-Glycine) and (b) tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate.

TABLE 1: Amino acids' profile of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate by HPLC.

	Amino acid	Retention time (min)	Concentration (mg/100 g)	Content (%)
1	Asp	5.56	10.62 ± 0.004	9.2 ± 0.04
2	Glu	6.63	6.55 ± 0.003	6.7 ± 0.03
3	H-Pro	10.79	0.29 ± 0.002	0.5 ± 0.02
4	Ser	14.40	2.79 ± 0.003	7.7 ± 0.03
5	Gly	15.55	12.44 ± 0.003	9.7 ± 0.02
6	His	17.19	0.87 ± 0.002	3.2 ± 0.02
7	Arg	19.11	14.34 ± 0.002	7.8 ± 0.02
8	Thr	19.62	4.45 ± 0.002	4.6 ± 0.02
9	Ala	19.83	5.83 ± 0.004	5.4 ± 0.04
10	Pro	20.38	9.47 ± 0.002	6.6 ± 0.02
11	Tyr	26.00	0.72 ± 0.003	2.2 ± 0.03
12	Val	27.22	1.97 ± 0.002	3.5 ± 0.02
13	Met	28.43	5.32 ± 0.003	5.6 ± 0.03
14	Ile	31.17	4.02 ± 0.035	2.3 ± 0.04
15	Leu	31.55	7.72 ± 0.002	5.1 ± 0.02
16	Phe	33.92	5.87 ± 0.002	3.1 ± 0.02
17	Lys	34.72	32.24 ± 0.003	17.0 ± 0.03

Data represent mean \pm standard deviation of three independent repeats.

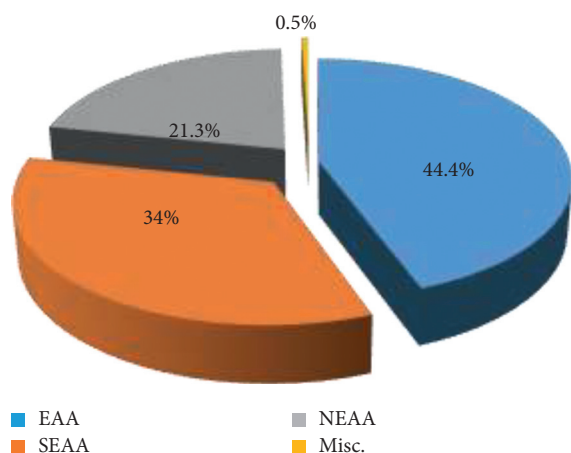


FIGURE 2: Essential amino acids (EAA), semiessential amino acids (SEAA), and nonessential amino acids (nEAA) of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate.

TABLE 2: Na, K, Mg, Ca, Mn, Zn, Fe, and Cu contents of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate.

Name	Concentration (mg/100 g)
Na	858.4 ± 0.02
K	778.5 ± 0.03
Mg	94.7 ± 0.02
Ca	2.1 ± 0.02
Mn	0.3 ± 0.03
Zn	0.6 ± 0.02
Fe	0.3 ± 0.03
Cu	0.1 ± 0.04

Data represent mean ± standard deviation of three independent repeats.

TABLE 3: Heavy metals of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate.

Name	LOQ	Concentration (μg/g)
Cd	0.3	0.013 ± 0.003
As	0.025	0.013 ± 0.002
Pb	4.2	36.35 ± 0.010
Hg	3	8.00 ± 0.001

Data represent mean ± standard deviation of three independent repeats.

3.4. FTIR. Infrared spectroscopy is a valuable analytical technique for obtaining rapid information about the structure and chemical changes of compounds during the different processes. The FTIR spectra of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate are presented in Figure 3. The spectrum had two low-intensity peaks at 1624 and 1728 cm^{-1} . The signal around 1624 cm^{-1} was due to the symmetric deformation vibration of adsorbed water molecules [33]. The peak at 1728 cm^{-1} was attributed to the C=O stretching vibration of formate ester moieties. The low intensity of this band suggested that only small amounts of this specific band are formed [34]. Tunichrome showed a strong O–H stretching absorption band around 3300 cm^{-1} and C–H stretching absorption band around 2962 cm^{-1} (Table 5). Similar results were reported by Núñez-Pons et al. [35] and Song et al. [36]. Moreover, these results

TABLE 4: Fluoride, chloride, bromide, nitrite, nitrate, phosphate, and sulfate contents of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate.

Name	RT (min)	Concentration (μg/g)
Fluoride	3.75	17.50 ± 0.2
Chloride	5.68	1750 ± 0.3
Bromide	8.59	15 ± 0.2
Nitrite	7.33	5 ± 0.1
Nitrate	10.39	850 ± 0.2
Phosphate	11.72	5 ± 0.1
Sulfate	14.41	15 ± 0.1

Data represent mean ± standard deviation of three independent repeats.

were in agreement with those obtained by GC-MS for the detection of carbocyclic, acidic, and steric groups.

3.5. Chemical Composition. The chemical composition of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate by GC-MS is reported in Table 6. Octadecanoic acid methyl ester (25.74%), nonanal (14.08%), hydrazinecarbothioamide (12.38%), octadecanoic acid (9.67%), and hexadecanoic acid (9.06%) were the main components extracted by methanol: hexane: chloroform (1 : 1 : 1). Similar components in tunichrome of a marine ascidian, *Lissoclinum bistratum*, were reported by Karthi et al. [37] after analysis by the GC-MS method.

This important analysis showed that tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate contained a large number of bioactive compounds which are known as antioxidant and antimicrobial natural products. Several amazing medicinal and pharmacological activities were also reported for tunichrome. According to the previous studies, some identified components such as 3-methyl-2-[4-(3-methyl-butoxy)-benzoylamino]-butyric acid [38], hexadecanoic acid [39], and phenol, 2,4-bis(1,1-dimethylethyl)- [40] exhibit high antioxidant activity.

The antimicrobial activities of oxazine [41], 1,3-oxazine [42], nonanal [43], hydrazinecarbothioamide [44], triazine [45], butyric acid [46, 47], (+)-trans-3,4-Dimethyl-2-phenyltetrahydro-1,4-thiazine [48–50], hexadecanoic acid [47], octadecanoic acid [51], spirost-8-en-11-one,3-hydroxy-, (3β, 5α, 14β.20β, 22β, 25R)- [52, 53], and phenol, 2,4-bis(1,1-dimethylethyl)- [40] were also well documented.

In addition, other pharmaceutical properties such as sedative, analgesic, antipyretic, anticonvulsant, antitubercular, antitumor, and antimalarial properties of 4-phenyltetrahydro-1,3-oxazine-2-thione [41], antitumor and anti-inflammatory properties of (+)-trans-3,4-dimethyl-2-phenyltetrahydro-1,4-thiazine [54], antitrypanosomal properties of hydrazinecarbothioamide, 2-[1-(4-nitrophenyl)ethylidene]- [55], and anticancer, antiproliferative, and anti-inflammatory properties of spirost-8-en-11-one,3-hydroxy-, (3β, 5α, 14β.20β, 22β, 25R)- [56, 57] were reported previously.

3.6. Antioxidant Properties. DPPH is a stable radical with an absorbance at 517 nm which can react with any antioxidant. This interaction can decrease the absorbance due to the color

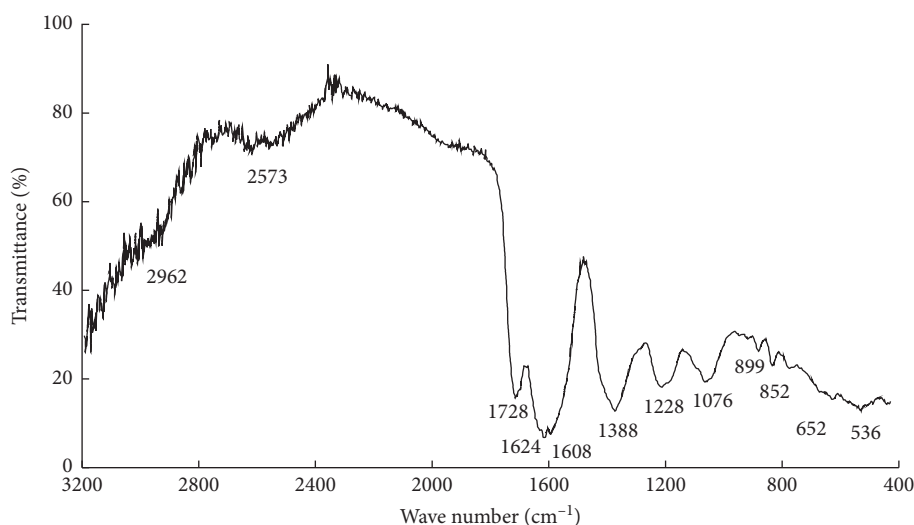


FIGURE 3: FTIR spectrum of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate.

TABLE 5: FTIR of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate.

Wavenumber (cm ⁻¹)		
1	2962	C-H stretching
2	2573	O-H stretch vibration
3	1728	C=O stretch vibration
4	1624	C=C symmetric deformation vibration
5	1608	N-O stretch vibration
6	1388	
7	1228	
8	1076	
9	899	
10	852	
11	652	
12	536	C-Br stretch vibration

change from purple to white or yellow [58, 59]. TPC and TFC of the sample were 0.55 mg GA/g and 0.21 mg quercetin/100 g, respectively. The RSA (%) values of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate and ascorbic acid are reported in Table 7. Tunichrome showed higher antioxidant activity than ascorbic acid, and RSA values were increased from 30.28 to 82.08% by increasing the concentration from 50 to 200 ppm, indicating its dose-dependent activity. These results showed that tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate can be an appropriate source of natural antioxidants for food and pharmaceutical purposes.

The antioxidant activities of 3-methyl-2-[4-(3-methylbutoxy)-benzoylamino]-butyric acid [38] and hexadecanoic acid [39] and phenol, 2,4-bis(1,1-dimethylethyl)- [40] were also reported previously. Lee et al. [15] evaluated the antioxidant activity of extracts of Stalked sea squirt *Styela clava* tunic. They reported that this extract showed high dose-dependent antioxidant activity, and water extract had 0.192 mg/ml. Also, Lee et al. [60] showed that the RSA of the water and ethanol *Styela clava* tunic extracts was 31 and 48.6% at 10 mg/ml, respectively. Lee et al. [61] also reported

high scavenging activities (50%) of starfish *Acanthaster planci* extracts at concentrations of 1.62 mg/ml, >10 mg/ml, and 4.03 mg/ml for the ethanol, ethyl acetate, and n-butanol extracts, respectively. Kim [14] reported that hydrolysates of solitary tunicate (*Styela clava*) had high antioxidant activity.

3.7. Antimicrobial Content

3.7.1. Well Diffusion Agar. Antimicrobial activity of tunichrome was reported by Sugumaran and Robinson [62] previously. Inhibition zones (mm) of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate (150 µg/ml) and gentamicin (10 µg/ml) are reported in Table 8. Inhibition zones of *Staphylococcus aureus*, *Staphylococcus aureus*, *Salmonella enterica*, and *Escherichia coli* O157:H7 were 14, 18, 17, and 15 mm, respectively. This sample had the highest antimicrobial activity against *Bacillus cereus*. Independent of bacterial strain, the antimicrobial activity of tunichrome was lower than gentamicin, as shown in Table 8. These results showed the significant antimicrobial activity of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate as a natural compound in food application.

The antimicrobial activities of oxazine and its derivatives were reported by Sindhu et al. [41] against *S. aureus* and *E. coli*. The antimicrobial activities of 1,3-oxazine against *Enterococcus faecalis* and *Listeria monocytogenes* were also reported by Hamza et al. [42]. The antimicrobial activity of nonanal was reported by Zhang et al. [43]. The antimicrobial potential of hydrazinecarbothioamide against *Bacillus subtilis*, *E. coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Paenibacillus macerans*, and *Salmonella typhimurium* was reported by Shim et al. [44]. Mohammadi [45] studied the antimicrobial activities of triazene against *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Micrococcus luteus*. The butyric acid activities against *Salmonella enterica*, *E. coli* and *Campylobacter jejuni* [46], *Candida albicans*, *Streptococcus mutans*, and *Streptococcus sanguinis*

TABLE 6: Chemical composition of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate by GC-MS.

	RT (min)	Name	Formula	MW (g/mol)	Content (%)
1	3.22	4-Phenyltetrahydro-1,3-oxazine-2-thione	C ₁₀ H ₁₁ NOS	193.27	0.71
2	5.92	Nonanal	C ₉ H ₁₈ O	142.24	14.08
3	8.39	3,3-Dimethyl-1-(2-carboxyphenyl)triazene	C ₉ H ₁₁ N ₃ O ₂	193.20	0.87
4	8.48	3-Methyl-2-[4-(3-methyl-butoxy)-benzoylamino]-butyric acid	C ₁₇ H ₂₅ NO ₄	307.40	0.87
5	8.68	p-Cynophenyl p-(2-propoxyethoxy)benzoat	C ₁₉ H ₁₉ NO ₄	325.40	0.87
6	8.88	(+)-trans-3,4-Dimethyl-2-phenyltetrahydro-1,4-thiazine	C ₁₂ H ₁₇ NS	207.30	0.87
7	17.06	Phenol, 2,4-bis(1,1-dimethylethyl)-	C ₁₄ H ₂₂ O	206.32	0.73
8	17.68	Hexadecanoic acid, methyl ester	C ₁₇ H ₃₄ O ₂	270.50	9.06
9	18.77	n-Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	256.42	5.84
10	21.96	Hydrazinecarbothioamide, 2-[1-(4-nitrophenyl)ethylidene]-	C ₉ H ₁₀ N ₄ O ₂ S	238.27	12.38
11	23.02	Octadecanoic acid, methyl ester	C ₁₉ H ₃₈ O ₂	298.50	25.74
12	24.02	Octadecanoic acid	C ₁₈ H ₃₆ O ₂	284.50	9.67
13	27.32	Spirost-8-en-11-one,3-hydroxy-, (3 β , 5 α , 14 β .20 β , 22 β , 25R)-	C ₂₇ H ₄₀ O ₄	428.00	0.87
14	29.37	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	C ₁₉ H ₃₈ O ₄	330.50	17.43

TABLE 7: RSA (%) of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate and ascorbic acid.

Concentration (ppm)	Tunichrome	Ascorbic acid
50	30.28 \pm 0.2Ca	17.86 \pm 0.1Db
100	47.59 \pm 0.1Ca	22.04 \pm 0.2Cb
150	66.28 \pm 0.2Ba	42.47 \pm 0.1Bb
200	82.08 \pm 0.1Aa	53.83 \pm 0.1Ab

Data represent mean \pm standard deviation of three independent repeats. Different capital letters in each column and lowercase ones in each row indicate significant differences ($P < 0.05$).

TABLE 8: Inhibition zones (mm) of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate (150 μ g/ml) and gentamicin (10 μ g/ml).

Bacteria	Tunichrome (mm)	Gentamicin (mm)
<i>Staphylococcus aureus</i>	14 \pm 0.3Db	16 \pm 0.2Da
<i>Bacillus cereus</i>	18 \pm 0.3Ab	19 \pm 0.2Aa
<i>Salmonella enterica</i>	17 \pm 0.2Bb	18 \pm 0.3Ba
<i>Escherichia coli</i> O157:H7	15 \pm 0.2Cb	17 \pm 0.3Ca

Data represent mean \pm standard deviation of three independent repeats. Different capital letters in each column and lowercase ones in each row indicate significant differences ($P < 0.05$).

TABLE 9: MIC and MBC values of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate.

Bacteria	MIC (mg/ml)	MBC (mg/ml)
<i>Staphylococcus aureus</i>	1.17 \pm 0.00Ab	2.34 \pm 0.00Aa
<i>Bacillus cereus</i>	0.59 \pm 0.00Bb	1.17 \pm 0.00Ba
<i>Salmonella enterica</i>	0.59 \pm 0.00Bb	1.17 \pm 0.00Ba
<i>Escherichia coli</i> O157:H7	1.17 \pm 0.00Ab	2.34 \pm 0.00Aa

Data represent mean \pm standard deviation of three independent repeats. Different capital letters in each column and lowercase ones in each row indicate significant differences ($P < 0.05$).

[47] were also documented. Moreover, the antimicrobial activities of (+)-trans-3,4-dimethyl-2-phenyltetrahydro-1,4-thiazine against *Mycobacterium tuberculosis* [48], *Staphylococcus aureus* and *E. coli* [49], *Staphylococcus aureus*, *E. coli*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* were reported previously [50].

The antimicrobial activities of hexadecanoic acid against *Candida albicans*, *Streptococcus mutans*, and *Streptococcus sanguinis* were shown by Huang et al. [47]. According to da Silva et al. [51], octadecanoic acid had significant antimicrobial activities against *Bacillus subtilis* and *Bacillus cereus*. The antimicrobial activities of spirost-8-en-11-one,3-hydroxy-, (3 β , 5 α , 14 β .20 β , 22 β , 25R)- against *E. coli*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* were reported in [52, 53]. The antimicrobial activities of phenol, 2,4-bis(1,1-dimethylethyl)- against *Aspergillus niger*, *Fusarium oxysporum*, and *Penicillium chrysogenum* were reported by Varsha et al. [40].

Galinier et al. [18] evaluated the two antimicrobial peptides from hemocytes of the *Halocynthia papillosa* tunicate. They reported that these components had high antimicrobial activities against *S. aureus* and *E. coli*. Cai et al. [5] reported that tunichromes showed antimicrobial activity against *E. coli* and *Photobacterium phosphoreum*.

3.7.2. MIC and MBC. Table 9 shows the MIC and MBC of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate. MIC values of tunichrome for *Staphylococcus aureus*, *Staphylococcus aureus*, *Salmonella enterica*, and *Escherichia coli* O157:H7 were 1.17, 0.59, 0.59, and 1.17 mg/ml, respectively. Moreover, the values of MBC for the *Staphylococcus aureus*, *Staphylococcus aureus*, *Salmonella enterica*, and *Escherichia coli* O157:H7 were 2.34, 1.17, 1.17, and 2.34 mg/ml, respectively. It was reported that styelins, clavanins, halocyanine, and plicatamide of tunichrome separated from ascidian blood cells exhibit antimicrobial properties [62, 63]. Cai et al. [5] also reported that there are different components in the tunichrome with *in vitro* antibiotic properties.

4. Conclusion

The aim of this study was to evaluate the physicochemical, nutraceutical, antioxidant, and antibacterial properties of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate. The results showed that this release contained a high amount of essential amino acids, especially

lysine. Also, the GC-MS profile showed that this compound included the functional, antioxidant, and antimicrobial components. Tunichrome had high antioxidant activity, TPC and TFC. Also, the strong antimicrobial activities against *Staphylococcus aureus*, *Staphylococcus aureus*, *Salmonella enterica*, and *Escherichia coli* O157:H7 were observed. Therefore, tunichrome can be considered as a good source of natural antioxidants and antimicrobials for future food and pharmaceutical applications.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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